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T-cell epitopes in erythropoietin

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T-CELL EPITOPES IN ERYTHROPOIETIN

FIELD OF THE INVENTION

The present invention relates to polypeptides to be administered especially to humans and in particular for therapeutic use. The polypeptides are modified polypeptides whereby the modification results in a reduced propensity for the polypeptide to elicit an immune response upon administration to the human subject. The invention in particular relates to the modification of erythropoietin (EPO) to result in EPO proteins that are substantially non-immunogenic or less immunogenic than any non-modified counterpart when used *in vivo*. The invention relates furthermore to T-cell epitope peptides derived from said non-modified protein by means of which it is possible to create modified EPO variants with reduced immunogenicity.

BACKGROUND OF THE INVENTION

There are many instances whereby the efficacy of a therapeutic protein is limited by an unwanted immune reaction to the therapeutic protein. Several mouse monoclonal antibodies have shown promise as therapies in a number of human disease settings but in certain cases have failed due to the induction of significant degrees of a human anti-murine antibody (HAMA) response [Schroff, R. W. et al (1985) *Cancer Res.* 45: 879-885; Shawler, D.L. et al (1985) *J. Immunol.* 135: 1530-1535]. For monoclonal antibodies, a number of techniques have been developed in attempt to reduce the HAMA response [WO 89/09622; EP 0239400; EP 0438310; WO 91/06667]. These recombinant DNA approaches have generally reduced the mouse genetic information in the final antibody construct whilst increasing the human genetic information in the final construct. Notwithstanding, the resultant "humanized" antibodies have, in several cases, still elicited an immune response in patients [Issacs J.D. (1990) *Sem. Immunol.* 2: 449, 456; Rebello, P.R. et al (1999) *Transplantation* 68: 1417-1420].

Antibodies are not the only class of polypeptide molecule administered as a therapeutic agent against which an immune response may be mounted. Even proteins of human origin and with the same amino acid sequences as occur within humans can still induce an immune response in humans. Notable

examples amongst others include the therapeutic use of granulocyte-macrophage colony stimulating factor [Wadhwa, M. et al (1999) *Clin. Cancer Res.* 5: 1353-1361] and interferon alpha 2 [Russo, D. et al (1996) *Bri. J. Haem.* 94: 300-305; Stein, R. et al (1988) *New Engl. J. Med.* 318: 1409-1413]. In such situations where these human proteins are immunogenic, there is a presumed breakage of immunological tolerance that would otherwise have been operating in these subjects to these proteins.

This situation is different where the human protein is being administered as a replacement therapy for example in a genetic disease where there is a constitutional lack of the protein such as can be the case for diseases such as hemophilia A, Christmas disease, Gauchers disease and numerous other examples. In such cases, the therapeutic replacement protein may function immunologically as a foreign molecule from the outset, and where the individuals are able to mount an immune response to the therapeutic, the efficacy of the therapy is likely to be significantly compromised.

Irrespective of whether the protein therapeutic is seen by the host immune system as a foreign molecule, or if an existing tolerance to the molecule is overcome, the mechanism of immune reactivity to the protein is the same. Key to the induction of an immune response is the presence within the protein of peptides that can stimulate the activity of T-cells via presentation on MHC class II molecules, so-called "T-cell epitopes". Such T-cell epitopes are commonly defined as any amino acid residue sequence with the ability to bind to MHC Class II molecules. Implicitly, a "T-cell epitope" means an epitope which when bound to MHC molecules can be recognized by a T-cell receptor (TCR), and which can, at least in principle, cause the activation of these T-cells by engaging a TCR to promote a T-cell response.

MHC Class II molecules are a group of highly polymorphic proteins which play a central role in helper T-cell selection and activation. The human leukocyte antigen group DR (HLA-DR) are the predominant isotype of this group of proteins however, isotypes HLA-DQ and HLA-DP perform similar functions. The present invention is applicable to the detection of T-cell epitopes presented within the

context of DR, DP or DQ MHC Class II. In the human population, individuals bear two to four DR alleles, two DQ and two DP alleles. The structure of a number of DR molecules has been solved and these appear as an open-ended peptide binding groove with a number of hydrophobic pockets which engage hydrophobic residues (pocket residues) of the peptide [Brown et al *Nature* (1993) 364: 33; Stern et al (1994) *Nature* 368: 215]. Polymorphism identifying the different allotypes of class II molecule contributes to a wide diversity of different binding surfaces for peptides within the peptide binding grove and at the population level ensures maximal flexibility with regard to the ability to recognise foreign proteins and mount an immune response to pathogenic organisms.

An immune response to a therapeutic protein proceeds via the MHC class II peptide presentation pathway. Here exogenous proteins are engulfed and processed for presentation in association with MHC class II molecules of the DR, DQ or DP type. MHC Class II molecules are expressed by professional antigen presenting cells (APCs), such as macrophages and dendritic cells amongst others. Engagement of a MHC class II peptide complex by a cognate T-cell receptor on the surface of the T-cell, together with the cross-binding of certain other co-receptors such as the CD4 molecule, can induce an activated state within the T-cell. Activation leads to the release of cytokines further activating other lymphocytes such as B cells to produce antibodies or activating T killer cells as a full cellular immune response.

T-cell epitope identification is the first step to epitope elimination, however there are few clear cases in the art where epitope identification and epitope removal are integrated into a single scheme. Thus WO98/52976 and WO00/34317 teach computational threading approaches to identifying polypeptide sequences with the potential to bind a sub-set of human MHC class II DR allotypes. In these teachings, predicted T-cell epitopes are removed by the use of judicious amino acid substitution within the protein of interest. However with this scheme and other computationally based procedures for epitope identification [Godkin, A.J. et al (1998) *J. Immunol.* 161: 850-858; Sturniolo, T. et al (1999) *Nat. Biotechnol.* 17: 555-561], peptides predicted to be able to bind MHC class II molecules may not function as T-cell epitopes in all situations, particularly, *in vivo* due to the

processing pathways or other phenomena. In addition, the computational approaches to T-cell epitope prediction have in general not been capable of predicting epitopes with DP or DQ restriction.

Equally, *in vitro* methods for measuring the ability of synthetic peptides to bind MHC class II molecules, for example using B-cell lines of defined MHC allotype as a source of MHC class II binding surface and may be applied to MHC class II ligand identification [Marshall K.W. et al. (1994) *J. Immunol.* 152:4946-4956; O'Sullivan et al (1990) *J. Immunol.* 145: 1799-1808; Robadey C. et al (1997) *J. Immunol* 159: 3238-3246]. However, such techniques are not adapted for the screening multiple potential epitopes to a wide diversity of MHC allotypes, nor can they confirm the ability of a binding peptide to function as a T-cell epitope.

Recently techniques exploiting soluble complexes of recombinant MHC molecules in combination with synthetic peptides have come into use [Kern, F. et al (1998) *Nature Medicine* 4:975-978; Kwok, W.W. et al (2001) *TRENDS in Immunol.* 22:583-588]. These reagents and procedures are used to identify the presence of T-cell clones from peripheral blood samples from human or experimental animal subjects that are able to bind particular MHC-peptide complexes and are not adapted for the screening multiple potential epitopes to a wide diversity of MHC allotypes.

Biological assays of T-cell activation remain the best practical option to providing a reading of the ability of a test peptide/protein sequence to evoke an immune response. Examples of this kind of approach include the work of Petra et al using T-cell proliferation assays to the bacterial protein staphylokinase, followed by epitope mapping using synthetic peptides to stimulate T-cell lines [Petra, A.M. et al (2002) *J. Immunol.* 168: 155-161]. Similarly, T-cell proliferation assays using synthetic peptides of the tetanus toxin protein have resulted in definition of immunodominant epitope regions of the toxin [Reece J.C. et al (1993) *J. Immunol.* 151: 6175-6184]. WO99/53038 discloses an approach whereby T-cell epitopes in a test protein may be determined using isolated sub-sets of human immune cells, promoting their differentiation *in vitro* and culture of the cells in the presence of synthetic peptides of interest and measurement of any induced

proliferation in the cultured T-cells. The same technique is also described by Stickler et al [Stickler, M.M. et al (2000) *J. Immunotherapy* 23:654-660], where in both instances the method is applied to the detection of T-cell epitopes within bacterial subtilisin. Such a technique requires careful application of cell isolation techniques and cell culture with multiple cytokine supplements to obtain the desired immune cell sub-sets (dendritic cells, CD4+ and or CD8+ T-cells) and is not conducive to rapid through-put screening using multiple donor samples.

As depicted above and as consequence thereof, it would be desirable to identify and to remove or at least to reduce T-cell epitopes from a given in principal therapeutically valuable but originally immunogenic peptide, polypeptide or protein. One of these therapeutically valuable molecules is erythropoietin (EPO). EPO is a glycoprotein hormone involved in the maturation of erythroid progenitor cells into erythrocytes. Naturally occurring EPO is produced by the liver during foetal life and by the kidney of adults. The hormone circulates in the blood to stimulate production of red blood cells in bone marrow. Anaemia is almost invariably a consequence of renal failure due to decreased production of EPO from the kidney. Recombinant EPO is used as an effective treatment of anaemia resulting from chronic renal failure. Recombinant human EPO (expressed in mammalian cells) having the 166 amino acid sequence depicted below [Jacobs, K. et al (1985) Nature, 313: 806-810; Lin, F.-K. et al (1985) Proc. Natl. Acad. Sci. U.S.A. 82:7580-7585] contains three N-linked and one O-linked oligosaccharide chains each containing terminal sialic acid residues. The latter are significant in enabling EPO to evade rapid clearance from the circulation by the hepatic asialoglycoprotein binding protein.

The mature amino acid sequence of human EPO (depicted in single-letter code) is as follows:

APPRLICDSRVLERYLLEAKEAENITTGCAEHCSLNENITVPDTKVNFYAWKRMEVGQQAVEVWQ
GLALLSEAVLRGQALLVNSSQPWEPLQLHVDKAVSGLRSLTTLLRALGAQKEAISPPDAASAAPL
RTITADTFRKLFRVYSNFLRGKLKLYTGEACRTGDR.

It is a particular objective of the present invention to provide modified EPO proteins in which the immune characteristic is modified by means of reduced numbers of potential T-cell epitopes.

Others have provided modified EPO molecules [US 5,856,298; US 5,955,422] but it is understood that these approaches have been directed towards improvements in the commercial production of EPO and approaches towards influencing the glycosylation status of the protein and particularly the recombinant form. These teachings do not recognise the importance of T cell epitopes to the immunogenic properties of the protein nor have been conceived to directly influence said properties in a specific and controlled way according to the scheme of the present invention.

It is highly desired to provide EPO with reduced or absent potential to induce an immune response in the human subject.

SUMMARY AND DESCRIPTION OF THE INVENTION

The present invention provides for modified forms of EPO, in which the immune characteristic is modified by means of reduced or removed numbers of potential T-cell epitopes.

The invention discloses sequences identified within the EPO primary sequence that are potential T-cell epitopes by virtue of MHC class II binding potential. This disclosure specifically pertains the human EPO protein of 166 amino acid residues.

The present invention discloses the major regions of the EPO primary sequence that are immunogenic in man and thereby provides the critical information required to conduct modification to the sequences to eliminate or reduce the immunogenic effectiveness of these sites.

In one embodiment, synthetic peptides comprising the immunogenic regions can be provided in pharmaceutical composition for the purpose of promoting a tolerogenic response to the whole molecule.

In a further embodiment EPO molecules modified within the epitope regions herein disclosed can be used in pharmaceutical compositions.

In summary the invention relates to the following issues:

- using a panel of synthetic peptides in a naïve T-cell assay to map the immunogenic region(s) of human EPO;
- using a panel of EPO protein variants in a naïve T-cell assay to select variants displaying minimal immunogenicity *in vitro*;
- using a panel of synthetic peptide variants in a naïve T-cell assay to select peptide sequences displaying minimal immunogenicity *in vitro*;
- using biological assays of T-cell stimulation to select a protein variant which exhibits a stimulation index of less than 2.0 and preferably less than 1.8 in a naïve T-cell assay;
- construction of a T-cell epitope map of EPO protein using PBMC isolated from healthy donors and a screening method involving the steps comprising: i) antigen priming *in vitro* using synthetic peptide or whole protein immunogen for a culture period of up to 7 days; ii) addition of IL-2 and culture for up to 3 days; iii) addition of primed T cells to autologous irradiated PBMC and re-challenge with antigen for a further culture period of 4 days and iv) measurement of proliferation index by any suitable method;
- EPO derived peptide sequences able to evoke a stimulation index of greater than 1.8 and preferably greater than 2.0 in a naïve T-cell assay and selected from any therapeutic protein;
- EPO derived peptide sequences selected from any therapeutic protein having a stimulation index of greater than 1.8 and preferably greater than 2.0 in a naïve T-cell assay wherein the peptide is modified to a minimum extent and tested in the naïve T-cell assay and found to have a stimulation index of less than 2.0;
- EPO derived peptide sequences sharing 100% amino acid identity with the wild-type protein sequence and able to evoke a stimulation index of 1.8 or greater and preferably greater than 2.0 in a T-cell assay;
- an accordingly specified EPO peptide sequence modified to contain less than 100% amino acid identity with the wild-type protein sequence and evoking a stimulation index of less than 2.0 when tested in a T-cell assay;
- an EPO molecule containing a modified peptide sequence which when individually tested evokes a stimulation index of less than 2.0 in a T-cell assay;

- an EPO molecule containing modifications such that when tested in a T-cell assay evokes a reduced stimulation index in comparision to a non modified protein molecule;
- an EPO molecule in which the immunogenic regions have been mapped using a T-cell assay and then modified such that upon re-testing in a T-cell assay the modified protein evokes a stimulation index smaller than the parental (non-modified) molecule and most preferably less than 2.0;
- a modified molecule having the biological activity of EPO and being substantially non-immunogenic or less immunogenic than any non-modified molecule having the same biological activity when used *in vivo*;
- an accordingly specified EPO molecule, wherein said loss of immunogenicity is achieved by removing one or more T-cell epitopes derived from the originally non-modified molecule;
- an accordingly specified EPO molecule, wherein said loss of immunogenicity is achieved by reduction in numbers of MHC allotypes able to bind peptides derived from said molecule;
- an accordingly specified EPO molecule, wherein one T-cell epitope is removed;
- an accordingly specified EPO molecule, wherein said originally present T-cell epitopes are MHC class II ligands or peptide sequences which show the ability to stimulate or bind T-cells via presentation on class II;
- an accordingly specified molecule, wherein 1 – 9 amino acid residues, preferably one amino acid residue in any of the originally present T-cell epitopes are altered;
- an accordingly specified molecule, wherein the alteration of the amino acid residues is substitution, addition or deletion of originally present amino acid(s) residue(s) by other amino acid residue(s) at specific position(s);
- an accordingly specified molecule, wherein, if necessary, additionally further alteration usually by substitution, addition or deletion of specific amino acid(s) is conducted to restore biological activity of said molecule;
- an accordingly specified molecule wherein alteration is conducted at one or more residues from the string of contiguous residues of sequence (a) RVLERYLLEAKEAENITTGCAEHCSLNENITVP and / or of sequence; (b) RGQALLVNSSQPWEPLQLHVDKAVSGLRSLTTL and / or of sequence; (c)

RTITADTFRKLFRVYSNFLRGKLKLYTGEACRT wherein said sequences are derived from the EPO wild-type sequence;

- an accordingly specified molecule where in addition to alteration conducted within one or more of the sequences (a), (b) or (c) above, alteration is conducted within any or all of the peptide sequences (i) – (iv) specified herein where peptide sequence (i) = MGVHECPAWLWLLLS; (ii) = VNFYAWKRMEVGQQA; (iii) = TTLLRALGAQKEASPPDAASA and (iv) = PDAASAAPLRTITAD;
- a peptide molecule comprising 13–15 consecutive residues from any of sequences (a), (b) or (c) above;
- a peptide molecule comprising at least 9 consecutive residues from any of the sequences (a), (b) or (c) above;
- a peptide molecule comprising 13–15 consecutive residues from any of sequences (i) – (iv) above;
- a peptide molecule comprising at least 9 consecutive residues from any of the sequences (i) – (iv) above;
- a peptide molecule of above sharing greater than 90% amino acid identity with any of the peptide sequences derived from (a), (b) or (c) above;
- a peptide molecule of above sharing greater than 80% amino acid identity with any of the peptide sequences derived from (a), (b) or (c) above;
- peptide sequences as above able to bind MHC class II;
- an accordingly specified EPO molecule, wherein one or more of the amino acid substitutions is conducted at a position corresponding to any of the amino acids specified within sequence (a) above;
- an accordingly specified EPO molecule, wherein one or more of the amino acid substitutions is conducted at a position corresponding to any of the amino acids specified within sequence (b) above;
- an accordingly specified EPO molecule, wherein one or more of the amino acid substitutions is conducted at a position corresponding to any of the amino acids specified within sequence (c) above;
- an accordingly specified EPO molecule, wherein one or more of the amino acid substitutions is conducted at a position corresponding to any of the amino acids specified within sequences (a), (b) or (c) above;
- a pharmaceutical composition comprising any of the peptides or modified peptides of above having the activity of binding to MHC class II

- a DNA sequence or molecule which codes for any of said specified modified molecules as defined above and below;
- a pharmaceutical composition comprising a modified molecule having the biological activity of EPO;
- a pharmaceutical composition as defined above and / or in the claims, optionally together with a pharmaceutically acceptable carrier, diluent or excipient;
- a method for manufacturing a modified molecule having the biological activity of EPO as defined in any of the claims of the above-cited claims comprising the following steps: (i) determining the amino acid sequence of the polypeptide or part thereof; (ii) identifying one or more potential T-cell epitopes within the amino acid sequence of the protein by any method including determination of the binding of the peptides to MHC molecules using *in vitro* or *in silico* techniques or biological assays; (iii) designing new sequence variants with one or more amino acids within the identified potential T-cell epitopes modified in such a way to substantially reduce or eliminate the activity of the T-cell epitope as determined by the binding of the peptides to MHC molecules using *in vitro* or *in silico* techniques or biological assays; (iv) constructing such sequence variants by recombinant DNA techniques and testing said variants in order to identify one or more variants with desirable properties; and (v) optionally repeating steps (ii) – (iv);
- an accordingly specified method, wherein step (iii) is carried out by substitution, addition or deletion of 1 – 9 amino acid residues in any of the originally present T-cell epitopes;
- an accordingly specified method, wherein the alteration is made with reference to an homologous protein sequence and / or *in silico* modelling techniques;
- a peptide sequence consisting of at least 9 consecutive amino acid residues of a 13mer T-cell epitope peptide as specified above and its use for the manufacture of EPO having substantially no or less immunogenicity than any non-modified molecule and having the biological activity of EPO when used *in vivo*;

The term "T-cell epitope" means according to the understanding of this invention an amino acid sequence which is able to bind MHC class II, able to stimulate T-

cells and / or also to bind (without necessarily measurably activating) T-cells in complex with MHC class II.

The term "peptide" as used herein and in the appended claims, is a compound that includes two or more amino acids. The amino acids are linked together by a peptide bond (defined herein below). There are 20 different naturally occurring amino acids involved in the biological production of peptides, and any number of them may be linked in any order to form a peptide chain or ring. The naturally occurring amino acids employed in the biological production of peptides all have the L-configuration. Synthetic peptides can be prepared employing conventional synthetic methods, utilizing L-amino acids, D-amino acids, or various combinations of amino acids of the two different configurations. Some peptides contain only a few amino acid units. Short peptides, e.g., having less than ten amino acid units, are sometimes referred to as "oligopeptides". Other peptides contain a large number of amino acid residues, e.g. up to 100 or more, and are referred to as "polypeptides". By convention, a "polypeptide" may be considered as any peptide chain containing three or more amino acids, whereas a "oligopeptide" is usually considered as a particular type of "short" polypeptide. Thus, as used herein, it is understood that any reference to a "polypeptide" also includes an oligopeptide. Further, any reference to a "peptide" includes polypeptides, oligopeptides, and proteins. Each different arrangement of amino acids forms different polypeptides or proteins. The number of polypeptides–and hence the number of different proteins–that can be formed is practically unlimited. "Alpha carbon (Cα)" is the carbon atom of the carbon-hydrogen (CH) component that is in the peptide chain. A "side chain" is a pendant group to Cα that can comprise a simple or complex group or moiety, having physical dimensions that can vary significantly compared to the dimensions of the peptide.

The invention may be applied to any EPO species of molecule with substantially the same primary amino acid sequences as those disclosed herein and would include therefore EPO molecules derived by genetic engineering means or other processes and may contain more or less than 193 amino acid residues.

EPO proteins such as identified from other mammalian sources have in common many of the peptide sequences of the present disclosure and have in common many peptide sequences with substantially the same sequence as those of the

disclosed listing. Such protein sequences equally therefore fall under the scope of the present invention.

The invention is conceived to overcome the practical reality that soluble proteins introduced with therapeutic intent in man trigger an immune response resulting in development of host antibodies that bind to the soluble protein. The present invention seeks to address this by providing EPO proteins with altered propensity to elicit an immune response on administration to the human host. According to the methods described herein, the inventors have discovered the regions of the EPO molecule comprising the critical T-cell epitopes driving the immune responses to this protein.

The general method of the present invention leading to the modified EPO comprises the following steps:
(a) determining the amino acid sequence of the polypeptide or part thereof;
(b) identifying one or more potential T-cell epitopes within the amino acid sequence of the protein by any method including determination of the binding of the peptides to MHC molecules using *in vitro* or *in silico* techniques or biological assays;
(c) designing new sequence variants with one or more amino acids within the identified potential T-cell epitopes modified in such a way to substantially reduce or eliminate the activity of the T-cell epitope as determined by the binding of the peptides to MHC molecules using *in vitro* or *in silico* techniques or biological assays. Such sequence variants are created in such a way to avoid creation of new potential T-cell epitopes by the sequence variations unless such new potential T-cell epitopes are, in turn, modified in such a way to substantially reduce or eliminate the activity of the T-cell epitope; and
(d) constructing such sequence variants by recombinant DNA techniques and testing said variants in order to identify one or more variants with desirable properties according to well known recombinant techniques.

The identification of potential T-cell epitopes according to step (b) can be carried out according to methods describes previously in the prior art. Suitable methods are disclosed in WO 98/59244; WO 98/52976; WO 00/34317 and may preferably

be used to identify binding propensity of EPO-derived peptides to an MHC class II molecule.

In practice a number of variant EPO proteins will be produced and tested for the desired immune and functional characteristic. The variant proteins will most preferably be produced by recombinant DNA techniques although other procedures including chemical synthesis of EPO fragments may be contemplated.

The invention relates to EPO analogues in which substitutions of at least one amino acid residue have been made at positions resulting in a substantial reduction in activity of or elimination of one or more potential T-cell epitopes from the protein. It is most preferred to provide EPO molecules in which amino acid modification (e.g. a substitution) is conducted within the most immunogenic regions of the parent molecule. The major preferred embodiments of the present invention comprise EPO molecules for which any of the MHC class II ligands are altered such as to eliminate binding or otherwise reduce the numbers of MHC allotypes to which the peptide can bind. The inventors herein have discovered immunogenic regions of the EPO molecule in man comprising the consecutive amino acid sequences; RVLERYLLEAKEAENITTGCAEHCSLNENITVP, RGQALLVNSSQPWEPLQLHVDKAVSGLRSLTTL and RTITADTFRKLFRVYSNFLRGKLKLYTGEACRT. Four additional peptide sequences may also be considered for alteration under the scheme of the present, such additional peptides having now been demonstrated to be capable of functioning as MHC class II ligands and stimulating T-cells according to recognised methods. The additional peptide sequences are:

i) MGVHECPAWLWLLLS
ii) VNFYAWKRMEVGQQA
iii) TTLLRALGAQKEASPPDAASA
iv) PDAASAAPLRTITAD

For the elimination of T-cell epitopes, amino acid substitutions are preferably made at appropriate points within the peptide sequence predicted to achieve substantial reduction or elimination of the activity of the T-cell epitope. In practice

an appropriate point will preferably equate to an amino acid residue binding within one of the pockets provided within the MHC class II binding groove.

It is most preferred to alter binding within the first pocket of the cleft at the so-called P1 or P1 anchor position of the peptide. The quality of binding interaction between the P1 anchor residue of the peptide and the first pocket of the MHC class II binding groove is recognized as being a major determinant of overall binding affinity for the whole peptide. An appropriate substitution at this position of the peptide will be for a residue less readily accommodated within the pocket, for example, substitution to a more hydrophilic residue. Amino acid residues in the peptide at positions equating to binding within other pocket regions within the MHC binding cleft are also considered and fall under the scope of the present.

It is understood that single amino acid substitutions within a given potential T-cell epitope are the most preferred route by which the epitope may be eliminated. Combinations of substitution within a single epitope may be contemplated and for example can be particularly appropriate where individually defined epitopes are in overlap with each other. Moreover, amino acid substitutions either singly within a given epitope or in combination within a single epitope may be made at positions not equating to the "pocket residues" with respect to the MHC class II binding groove, but at any point within the peptide sequence. Substitutions may be made with reference to an homologues structure or structural method produced using *in silico* techniques known in the art and may be based on known structural features of the molecule according to this invention. All such substitutions fall within the scope of the present invention.

Amino acid substitutions other than within the peptides identified above may be contemplated particularly when made in combination with substitution(s) made within a listed peptide. For example a change may be contemplated to restore structure or biological activity of the variant molecule. Such compensatory changes and changes to include deletion or addition of particular amino acid residues from the EPO polypeptide resulting in a variant with desired activity and in combination with changes in any of the disclosed peptides fall under the scope of the present.

In as far as this invention relates to modified EPO, compositions containing such modified EPO proteins or fragments of modified EPO proteins and related compositions should be considered within the scope of the invention. In another aspect, the present invention relates to nucleic acids encoding modified EPO entities. In a further aspect the present invention relates to methods for therapeutic treatment of humans using the modified EPO proteins. In this aspect the modified EPO protein may be linked with an antibody molecule or fragment of an antibody molecule. The linkage may be by means of a chemical cross-linker or the EPO-antibody moiety may be produced as a recombinant fusion protein.

In a further aspect still, the invention relates to methods for therapeutic treatment using pharmaceutical preparations comprising peptide or derivative molecules with sequence identity or part identity with the sequences herein disclosed.

PATENT CLAIMS:

1. An EPO molecule containing one or more amino acid modifications such that when tested in a T-cell assay evokes a reduced stimulation index in an individual in vivo in comparision to a non modified protein molecule in the same individual.

2. An EPO molecule of claim 1 containing a modified peptide sequence which when tested in an individual evokes a reduced immunogenicity measured by a stimulation index of less than 2.0 in a T-cell assay.

3. An EPO molecule according to claim 1 or 2, wherein said loss of immunogenicity is achieved by removing one or more T-cell epitopes derived from the originally non-modified molecule.

4. An EPO molecule of claim 3, wherein one T-cell epitope is removed;

5. An EPO molecule of claim 3 or 4, wherein said loss of immunogenicity is achieved by reduction in numbers of MHC allotypes able to bind peptides derived from said molecule.

6. An EPO molecule according to any of the claims 1 to 5, wherein said originally present T-cell epitopes are MHC class II ligands or peptide sequences which show the ability to stimulate or bind T-cells via presentation on class II.

7. An EPO molecule of claim 6, wherein 1 – 9 amino acid residues, preferably one amino acid residue in any of the originally present T-cell epitopes are altered.

8. An EPO molecule of claim 7, wherein the alteration of the amino acid residues is substitution, addition or deletion of originally present amino acid(s) residue(s) by other amino acid residue(s) at specific position(s).

9. An EPO molecule of claim 7 or 8, wherein alteration is conducted at one or more residues from the string of contiguous residues of the EPO wild-type sequence sequence

(a) RVLERYLLEAKEAENITTGCAEHCSLNENITVP,

(b) RGQALLVNSSQPWEPLQLHVDKAVSGLRSLTTL,

(c) RTITADTFRKLFRVYSNFLRGKLKLYTGEACRT.

10. An EPO molecule of claim 9, wherein in addition to alteration conducted within one or more of the sequences (a), (b) or (c), alteration is conducted within any or all of the peptide sequences (i) – (iv) specified herein, wherein peptide sequence (i) = MGVHECPAWLWLLS; (ii) = VNFYAWKRMEVGQQA; (iii) = TTLLRALGAQKEASPPDAASA and (iv) = PDAASAAPLRTITAD.

11. A T-cell epitope peptide comprising 13–15 consecutive residues from any of sequences (a) or (b) of claim 9 or 10.

12. A T-cell epitope peptide of claim 11 comprising at least 9 consecutive residues from any of the sequences (a), (b) or (c).

13. A T-cell epitope peptide of claim 11 or 12 sharing greater than 90% amino acid identity with any of the peptide sequences derived from (a), (b) or (c).

14. A T-cell epitope peptide of claim 11 or 12 sharing greater than 80% amino acid identity with any of the peptide sequences derived from (a), (b) or (c).

15. A T-cell epitope peptide of any of the claims 11 to 14 which is able to bind MHC class II.

16. An EPO derived peptide sequence according to any of the claims 11 to 15 which is able to evoke a stimulation index of greater than 1.8 and preferably greater than 2.0 in a naïve T-cell assay and selected from any therapeutic protein.

17. A peptide deriving from a peptide of claim 16, wherein the peptide is modified to a minimum extent by exchange of one or more amino acid residues having a stimulation index of less than 2.0 in a T-cell assay.

18. A DNA sequence or molecule which codes for any of said EPO molecules and T-cell epitopes as specified in any of the claims 1 to 17.

19. A pharmaceutical composition comprising any of said EPO molecules and T-cell epitopes as specified in any of the claims 1 to 18 and having the activity of binding to MHC class II together with a pharmaceutically acceptable carrier, diluent or excipient.

20. Use of a peptide sequence according to any of the claims 11 to 17 for the manufacture of EPO having substantially no or less immunogenicity in an individual than any non-modified molecule having the biological activity of EPO when used *in vivo* in the same individual.

21. A method for manufacturing a modified molecule having the biological activity of EPO as defined in any of the claims of the above-cited claims comprising the following steps: (i) determining the amino acid sequence of the polypeptide or part thereof; (ii) identifying one or more potential T-cell epitope peptides within the amino acid sequence of the protein by any method including determination of the binding of the peptides to MHC molecules using *in vitro* or *in silico* techniques or biological assays; (iii) designing new sequence variants with one or more amino acids within the identified potential T-cell epitopes modified in such a way to substantially reduce or eliminate the activity of the T-cell epitope as determined by the binding of the peptides to MHC molecules using *in vitro* or *in silico* techniques or biological assays; (iv) constructing such sequence variants by recombinant DNA techniques and testing said variants in order to identify one or more variants with desirable properties; and (v) optionally repeating steps (ii) – (iv), wherein step (ii) is carried out by one or more of the following sub-steps: (a) using a panel of synthetic peptides in a naïve T-cell assay to map the immunogenic region(s) of human EPO;

(b) using a panel of EPO protein variants in a naïve T-cell assay to select variants displaying minimal immunogenicity *in vitro*;

(c) using a panel of synthetic peptide variants in a naïve T-cell assay to select peptide sequences displaying minimal immunogenicity *in vitro*;

(d) using biological assays of T-cell stimulation to select a protein variant which exhibits a stimulation index of less than 2.0 and preferably less than 1.8 in a naïve T-cell assay;

(e) construction of a T-cell epitope map of EPO protein using PBMC isolated from healthy donors and a screening method involving the steps comprising: (1) antigen priming *in vitro* using synthetic peptide or whole protein immunogen for a culture period of up to 7 days; 2) addition of IL-2 and culture for up to 3 days; 3) addition of primed T cells to autologous irradiated PBMC and re-challenge with antigen for a further culture period of 4 days and 4) measurement of proliferation index by any suitable method;

22. The method of claim 21, wherein step (iii) is carried out by substitution, addition or deletion of 1 – 9 amino acid residues in any of the originally present T-cell epitopes;

23. The method of claim 21 or 22, wherein the alteration is made with reference to an homologous protein sequence and / or *in silico* modelling techniques,

ABSTRACT:

The present invention relates to polypeptides to be administered especially to humans and in particular for therapeutic use. The polypeptides are modified polypeptides whereby the modification results in a reduced propensity for the polypeptide to elicit an immune response upon administration to the human subject. The invention in particular relates to the modification of erythropoietin (EPO) to result in EPO proteins that are substantially non-immunogenic or less immunogenic than any non-modified counterpart when used *in vivo*. The invention relates furthermore to T-cell epitope peptides derived from said non-modified protein by means of which it is possible to create modified EPO variants with reduced immunogenicity.